AD_____

Award Number: DAMD17-98-1-8510

TITLE: Mechanisms of Mechano-Transduction within Osteoblasts

PRINCIPAL INVESTIGATOR: Louis C. Gerstenfeld, Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, Massachusetts 02118

REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (1 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
Mechanisms of Mechano-Transduction within Osteoblasts

5. FUNDING NUMBERS
DAMD17-98-1-8510

6. AUTHOR(S)
Louis C. Gerstenfeld, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University
Boston, Massachusetts 02118

E-MAIL:
lgersten@bu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
Mechanical stimulation is crucial to the homeostasis of adult bone density and mass. The hypothesis of this proposal is that bone cells sense their mechanical environment through specific cell surface receptors (integrins) that interact with specific extracellular matrix (ECM) proteins (osteopontin, bone sialoprotein, and fibronectin) that are the ligands for these receptors. We propose that the expression of these proteins is regulated in response to both cellular interactions with the ECM and mechanical stimulation. Thus, these proteins act like autocrine factors that modify cell behavior in response to changes in either matrix composition or mechanical deformation of the ECM itself. The proposed experiments will define how osteoblasts discriminate the molecular mechanisms by which mechanical signals mediat thier actions through the cellular interactions of integrins with the ECM. A determination of the specific integrin isotypes that are involved in the mechano-signal transduction process will be made. The signal transduction system(s) that are responsible for mediating osteopontin, bone sialoprotein and fibronectin gene expression in response to mechanical stimulation, will be determined. Other experiments will examine how aspects of the mechanical stimuli, such as frequency, intensity or duration effect cell response. Knowledge gained from understanding mechano-signal transduction will facilitate the development of appropriate clinical approaches to enhance the adaptive responses of skeletal tissue to mechanical stimulation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
osteoblast, mechanical stimulation, signal transduction, genomic regulation	15
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

INTRODUCTION

The bone remodeling cycle is known to be intimately involved in the metabolic homeostasis of mineral balance.[1] Bone formation and the remodeling cycle have been shown to be essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected.[2,3] Bone remodeling has also been hypothesized to provide the means of repairing bone tissue that has been damaged as a result of mechanical fatigue.[4] Thus, it may be speculated that the skeletal cells (osteoblasts and osteoclasts) which mediate the remodeling process are regulated by their mechanical environment. In order for osteoblasts to respond to their mechanical environment, they must in some way sense it. One mechanism by which cells sense mechanical signals is through the physical deformation of the tissue. The hypothesis of this proposal is that specific cell surface receptors (integrins) that interact with specific extracellular matrix proteins (collagen, osteopontin, bone sialoprotein, and fibronectin) provide the physical link through which mechanical stimuli are transmitted via tissue deformation. As a corollary to this hypothesis we propose that the extracellular proteins that are ligands for these receptors are themselves regulated in response to cellular interaction with both the matrix and mechanical stimulation. Thus, these proteins act both like autocrine factors that modify cell behavior in response to changes in matrix composition and mechanical deformation, as well as having a structural role in the matrix itself. Maintaining the balance of the extracellular matrix composition within bone then provides a mechanism by which the structural homeostasis of the skeleton may be regulated. This hypothesis and the published data that supports it are found in our review article published in 1999. [5]

The experiments that are being carried out with the support of this grant are designed to define how osteoblasts discriminate at a molecular level, and how mechanical stimuli and cellular interactions with the extracellular matrix are transduced through integrin receptors and converted to intracellular signals that lead to genomic changes. These experiments define whether different integrin ligands use common mechanisms in the regulation of their response to these stimuli. A determination of the intracellular second signaling systems (Kinases) that are responsible for mediating the altered gene expression of opn, bsp and fn to mechanical stimulation are being determined, both by assessing which kinases are activated and through the use of specific inhibitors that block the actions of the different kinase systems. A determination of the specific integrin isotypes that are involved in the different signal transduction processes has been made. Other experiments are examining if individual gene responses are differentially sensitive to various aspects of the mechanical stimuli (intensity, frequency, duration) or have different thresholds of response to different component parts of the stimuli.

BODY (Goals as Defined in Original Proposal)

Goal 1. The first goal of this proposal is directed at defining the molecular mechanisms of signal transduction by which mechanical stimulation regulates the expression of three specific ECM genes (osteopontin, bone sialoprotein and fibronectin) within osteoblasts. These studies will test whether there are common signal transduction pathways that mediate changes in the expression of these genes in response to mechanical stimulation.

a) The involvement of specific integrin isotypes in the mechano-signal transduction process that mediates these gene's genomic responses to mechanical stimulation will be assessed. Year 1

b) The mechanistic relationship between the signal transduction pathways that are activated by mechanical stimulation vs. cell adhesion will be examined years 2-3
c) The nature of the second signal transduction pathways that mediate the changes in expression for each of these genes will be determined. Years 2-3

The effect of cell adhesion on the expression of these genes was separately examined to determine if this stimuli would mimic the effects of the mechanical stimulation. All three genes also showed comparable levels of induction in response to adhesion on the fibronectin coated surfaces in comparison to non-coated surfaces with maximal levels of induction seen for *bsp* and *opn* at 24 hours after plating while *fn* showed maximal levels of stimulation at 8 hours. Interestingly, while both *opn* and *fn* mRNA expression returned to base line after cell adhesion on fibronectin, *bsp* mRNA levels remained elevated. Examination of the signal transduction pathways that mediated the gene expression in response to attachment on fibronectin coated surfaces showed that both genistein and cycloheximide inhibited the induction of all three genes. This demonstrates that a tyrosine kinase was involved in the cell attachment mediated induction of these genes, and new protein synthesis was a prerequisite to this process. In contrast, the PKA specific inhibitor H-89 only ablated the induction of *fn* expression. Depolymerization of either microtubules or microfilaments with colchicine or cytochalasin D respectively had little effect on the over all expression of these genes in response to cell adhesion, indicating that the adhesion mediated phenomena was not dependent on cytoskeletal integrity. In summary, these results show that both mechanical stimulation and cell adhesion specifically stimulated the expression of integrin binding proteins. These results further demonstrate that while there are common features in the signal transduction processes that mediated the induction of these genes, each gene was separately induced by unique mechanisms. **A complete description of these results is in a manuscript that was submitted and is now in revision for publication.** Carvalho RS, Schaffer JL, Bumann A. and Gerstenfeld LC. RGD containing proteins of osteoblasts are responsive to mechanical stimulation and matrix attachment. (submitted in 1999 and under revision)

In our second study, we examined three interrelated relationships pertaining to osteoblast adhesion and the transduction of molecular signals. This study is described completely below. The first aspect of this study was to define if osteoblasts have variable adhesion properties to different integrin ligands. The second component of the study was to define the relationship between the selective adhesion of the osteoblasts on the different ligands with the induction of specific second signal kinase activities. The final aspect of this study was to determine if osteoblat adhesion to specific integrin ligands would show specific induction of *opn* mRNA expression. The first series of experiments of this study are depicted in Figure 1, Panels A and B. These data show that three different extracellular matrix proteins, fibronectin (FN), fibrillar collagen type I (C), and denatured collagen type I gelatin (G), promote selective adhesion, whereas three other proteins, laminin (LN), osteopontin (OPN), and vitronectin (VN), did not. Bovine serum albumin (Al) was used as a control and is included in Panel B. As can be seen from this study, fibronectin was the most effective protein at promoting selective adhesion. Both native and denatured type I collagen also promoted selective attachment, but unlike fibronectin, both native and denatured collagen showed saturation at between 1 and 3$\mu g/cm^2$ of surface coating. In contrast, fibronectin did not show saturation in promoting cell adhesion until about 30$\mu g/cm^2$(data not shown). These data demonstrate that osteoblast adhesion is specifically

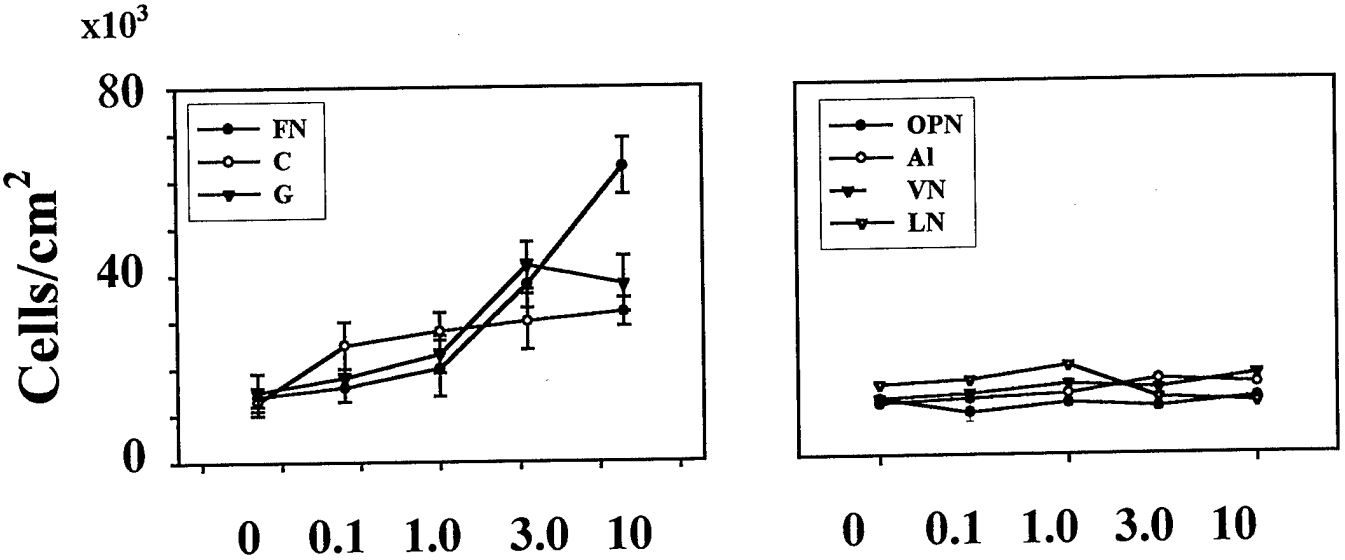

Figure 1. Relationship of ligand concentration to cell attachment. Bacterial petri dishes were coated with 0, 0.1, 1.0, 30 and 10 μg/cm^2 of each of the proteins. G=gelatin, denatured collagen, VN=vironectin, FN=fibronectin, OP=osteopontin, Al=bovine serum albumin, C=fibrillar collagen, native collagen, PL=polyL-lysine. Attachment was for 4 hours. Values were calculated as the total number of cells attached per cm^2 of coated surface area.

promoted by β1 but not by β3 containing integrins. They also suggest that there is a further discrimination between the individual integrin ligands since laminin did not strongly mediate cell adhesion.

The relationship of cell adhesion to the induction of specific intracellular kinase activities was next examined. (Figure 2 Panels A and B). In this study cell adhesion was carried out at a fixed concentration of coating (10 μg/cm^2) for one hour. These results are depicted in Figure 2, Panel A. Profiles of selectively mediated attachment of the embryonic osteoblasts was seen after only 60 minutes, comparable to the profiles seen in Figure 1, which had assessed attachment after four hours. The selective induction of two classes of kinases (PKA and PKC) that are involved in second signaling were then determined in parallel cultures that were identically prepared. It is interesting to note that attachment to fibronectin showed induction of both PKA and PKC activities, while native collagen showed the strongest induction of PKA with no induction seen for PKC. In contrast, while osteopontin did not promote cell adhesion, it did stimulate PKA activity, while denatured collagen and laminin both strongly inhibited PKA activity. These results show that specific effects on second signal kinase activities are dependent on the specific ligand interactions and not the processes of cell adhesion. Indeed, the strong inhibitory effect of denatured collagen on both PKA and PKC kinase activities while at the same time promoting cell adhesion, suggests that the biological processes that are mediated by these ligands are related to the structural conformation of these ligands and their interactions with specific receptors. Thus the processes of cellular adhesion most likely can be mediated solely through the RGD or other small integrin binding motiffs, but the down stream activation of intracellular signaling responses are specifically related to the structural conformations of the intact ligands .

The specific effects of fibronectin mediated cell adhesion on three structural and enzymatic components found with focal adhesion complexes were next assessed (Figure 3). In these studies the

intracellular distribution and quantities of focal adhesion kinase, pTyrosine and vinculin, were examined after adhesion on surfaces coated with either polyL-lysine or fibronectin. These data showed that adhesion to surfaces coated with either molecule did not appear to either alter the general intracellular localization or quantities of focal adhesion kinase or vinculin within an hour period. On the other hand

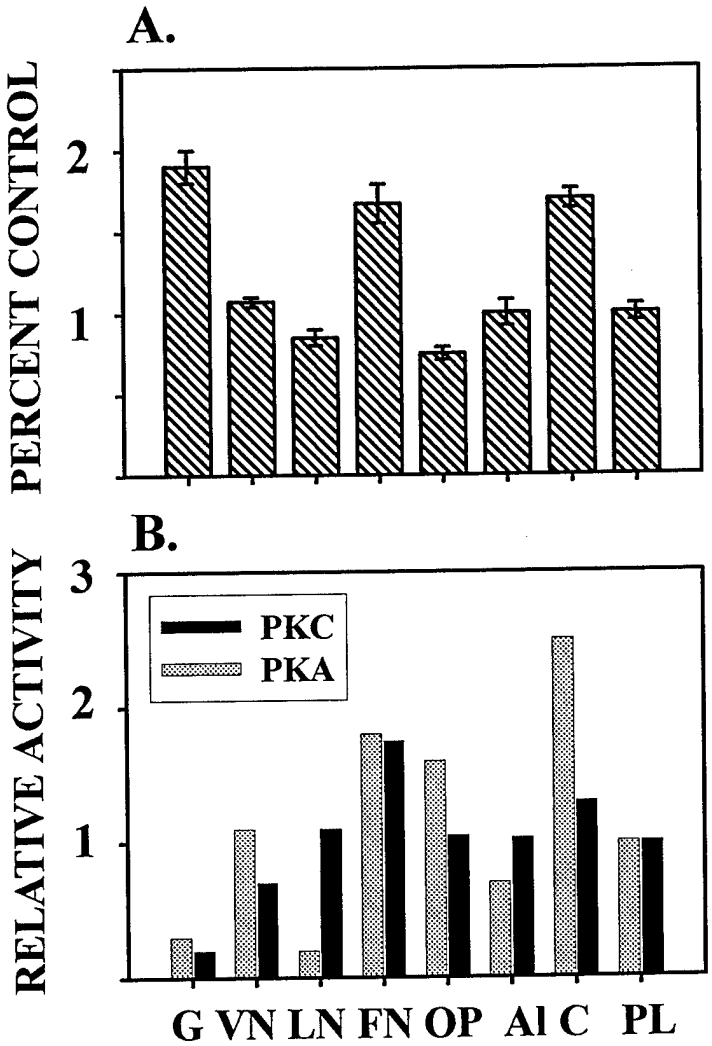

Figure 2 Relationship of Ligand Mediated Adhesion and Second Signal Kinase Activity. **Panel A**. Percent osteoblast attachment on selective protein coating. Petri dishes were coated with 2 $\mu g/cm^2$ of each of the proteins. G=gelatin, denatured collagen, VN=vironectin, FN=fibronectin, OP=osteopontin, Al=albumin, C=fibrillar collagen, native collagen, PL=polyL-lysine. Attachment was for 1 hours. Values were calculated as the percentage of cells on the experimental surfaces relative to the cell numbers observed on the polyL-lysine coated dishes.

Panel B Kinase activity after adhesion on selective protein coatings. General PKA and PKC activities were measured using specific fluorescent substrates. Enzyme activities were first normalized per total protein content used in each assay. Values were then calculated as the percentage of activities seen in the cells plated on the polyL-lysine coated dishes.

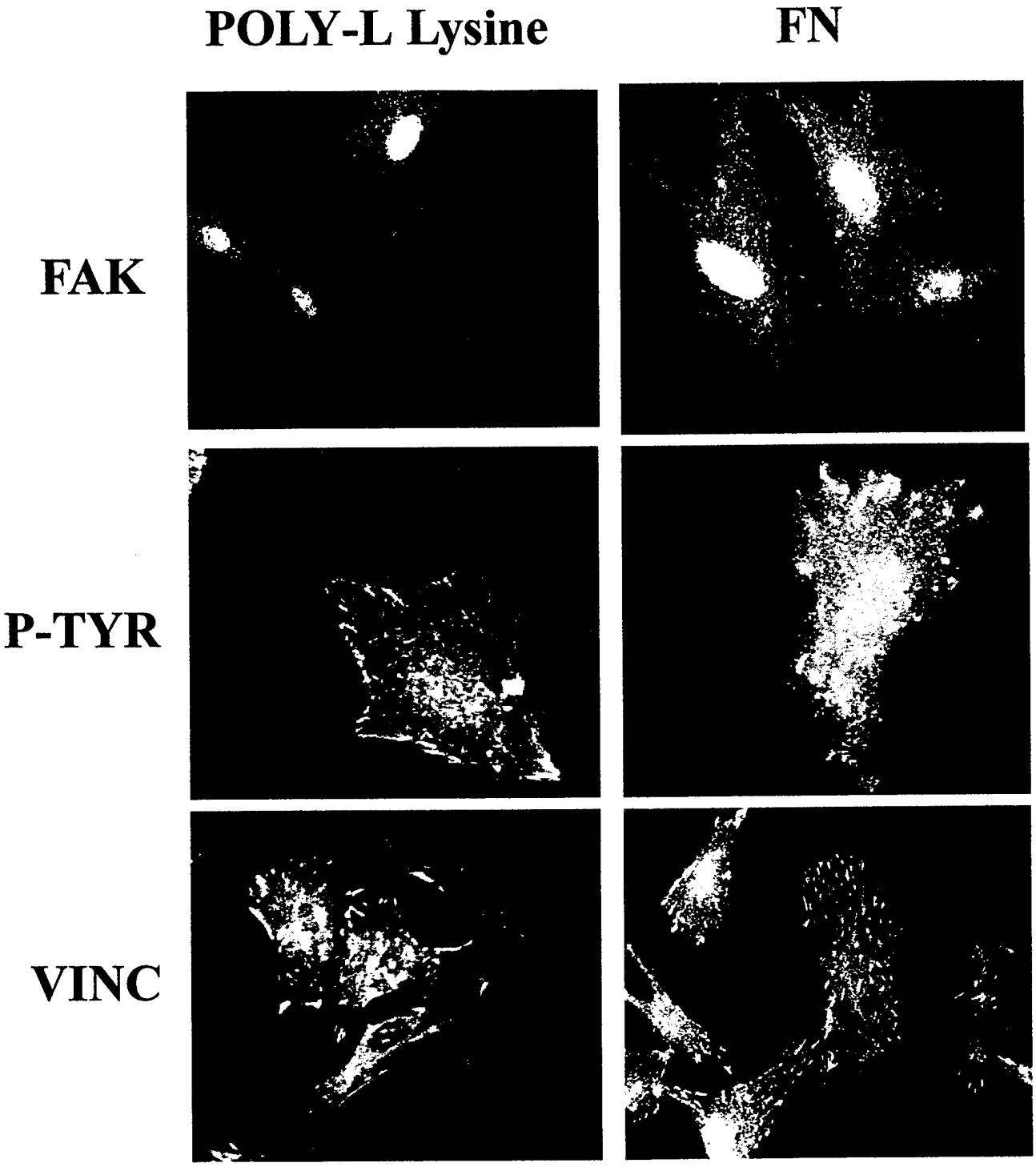

Figure 3. Intracellular response to adhesion on fibronectin. Cells were allowed to adhere to either polyL-lysine or fibronectin for 4 hours. The intracellular localization of focal adhesion kinase(FAK), P-Tyrosine,(Ptyr) and vinculin (VINC) were visualized by immunocytochemical analysis with selective antibodies to each of the three molecules.

there was a strong induction of p-tyrosine levels and a generalization of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand. These data then re-enforce the conclusions reached in the experiments depicted in figure two that suggest that the induction of intracellular kinase activities are related to the specific nature of the ligand's interactions with its receptor and less with the process of cellular adhesion alone.

The last set of experiments of this study examines the relationship between osteoblast adhesion on specific ligands and the induction of specific genomic changes, using osteopontin mRNA levels as a marker of genomic regulation. These studies are depicted in figure 4. As can be seen in this figure, the induction of increased osteopontin mRNA expression was seen as early as four hours after the cells had been allowed to adhere and continued to increase up to 24 hours. It is interesting to note in this study that once again native fibrillar collagen, fibronectin and to a lesser extent laminin induced osteopontin gene expression to increase. These results suggest that the same integrin receptors that facilitate specific cell attachment also facilitate the induction of osteopontin expression. It is also interesting to note that neither osteopontin nor vitronectin mediated either of these cell responses, suggesting that they are not facilitated through a $\alpha_v\beta_3$ receptor. The one difference that was observed, however, was in the comparison of cell attachment on denatured collagen vs. the induction of osteopontin. This result again suggests that signal transduction through the collagen receptor is only mediated when it interacts with native collagen.

Goal 2 The second goal of these studies will determine the relative importance of each component part of the mechanical stimuli (intensity, frequency and duration) to the mechano-signal transduction process.

a) The component of the mechanical stimuli (intensity, frequency, and duration) that is responsible for producing the signal transduction, which leads to the genomic regulation of specific genes in osteoblasts, will be examined. Years 1-2
b) Short term (<24hr) vs. chronic (>72hr) adaptive responses of osteoblasts to variations in duration of mechanical perturbation will be defined. Years 3

Towards the completion of goal two, initial studies had been carried out last year by a masters student, Emily Samuels, to determine the component part of mechanical stimuli that would induce changes in osteopontin gene expression. In these preliminary studies we analyzed duration and frequency of the mechanical stimulation and examined the effect of fluid flow-induced shear stress on osteopontin expression. Using our device that delivers spatially uniform biaxial strain to a membrane surface, osteoblasts were subjected to 1% strain for 1,5,15,30 minutes and 1,2,4,and 8 hours of strain. Analysis of the induction of osteopontin mRNA expression demonstrated that a maximal 145% induction was observed after 8 hours. ($p.<0.05$). Osteoblasts were then subjected to varying frequencies of strain (0.1, 0.25, 0.5 and 1 Hz) for 8 hours. There was clearly a 2-3 fold stimulation in response to increasing frequency again with a significant finding ($p<0.05$). The effect of fluid induced shear stress was examined in the third part of this study. This was accomplished in the following manner. The membrane was cut into two equal areas that encompassed the inner and outer circular areas of the membrane. The cells were then subjected to an 8 hour period of mechanical stimulation at the same variations in frequency as used in the first part of these studies. Because of the way the device generates its strains the inner circular area has several fold lower fluid flow than the outer areas. Similarly

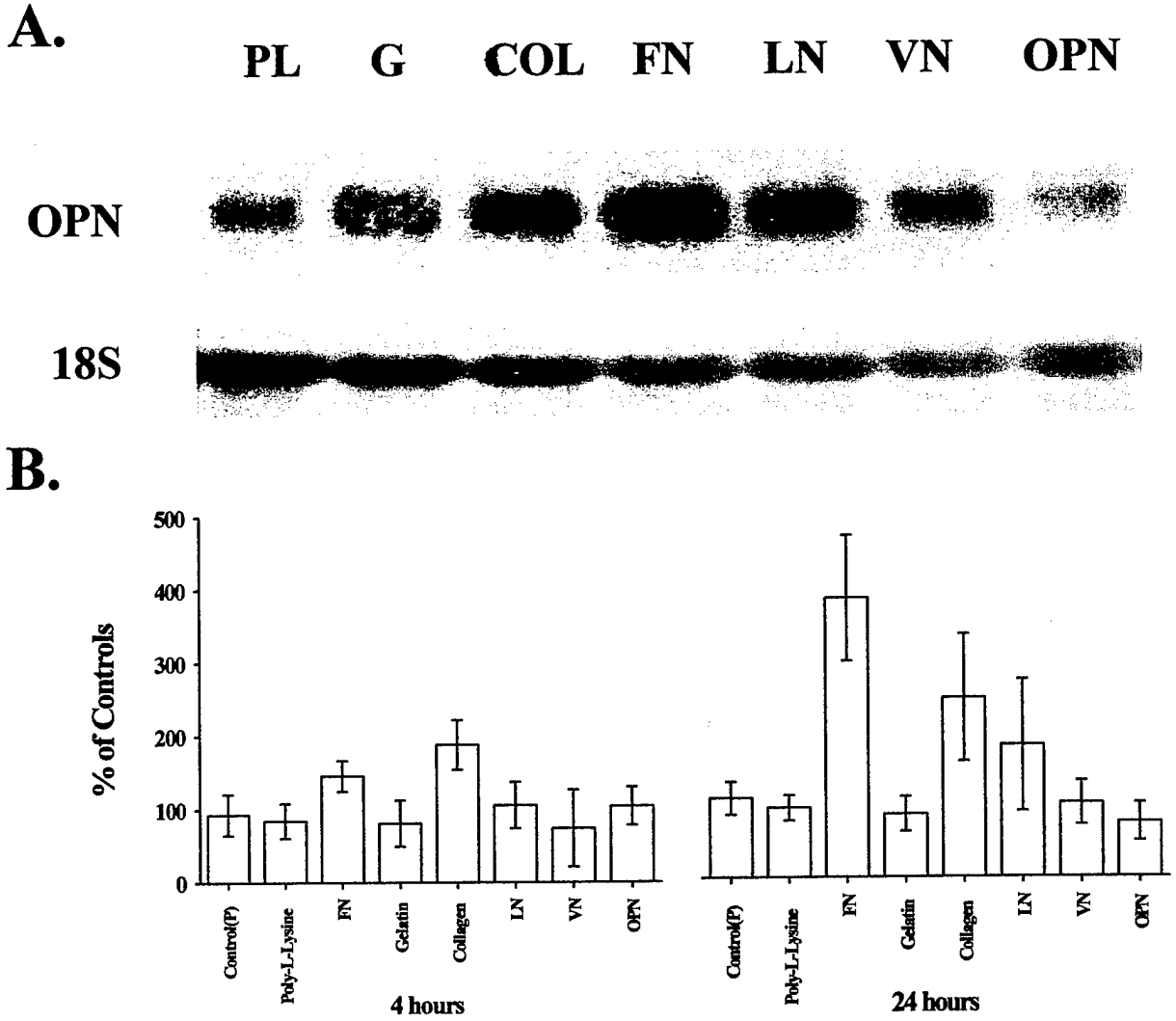

Figure 4 Induction of osteopontin expression in response to cell attachment on various ECM proteins. **Panel A**: Osteopontin expression was measured by Northern blot analysis and the band intensities were normalized to the 18S band. PL=polylysine, G=gelatin, denatured collagen, COL=native fibrillar collagen, FN= fibronection, LN=laminin, VN=vitronectin, OPN=osteopontin. **Panel B:** Graphical measurement of osteopontin mRNA induction in response to cell adhesion. Left panel shows percent induction after four hours and right panel after 24 hours. The nature of the protein coatings are denoted in the figure Error bar represents the S.E. of northern blots performed on mRNAs from experiments performed on three separate sets of cells.

higher frequencies will generate higher fluid flows, thus the combination of inner and outer areas with increasing frequency of strain should produce several orders of magnitude greater fluid movement. Analysis of osteopontin mRNA production, however, showed that only the highest frequency (1 Hz) generated altered levels of osteopontin expression when comparing the inner and outer areas of the membrane. These results also showed that it was inversely related to the levels of fluid flow. Thus, the inner areas had the higher levels of osteopontin induction. These results were completely opposite to the hypothesis that fluid shear stress was the major mechanical stimulation that facilitated osteopontin expression. These results also would lead to questions as to whether Ca flux through stress activated channels was a component part of the signal transduction mechanisms that induced osteopontin expression as has been suggested by other research groups.[6] **We are currently repeating all of these initial studies to verify these results.**

KEY RESEARCH ACCOMPLISHMENTS

1. We have completed almost all of the experiments outlined in goal one.
2. We have partially completed the components of goal two and are currently repeating a number of experiments that we have initiated in year one.

Reportable Outcomes

Manuscripts, Abstracts, and Presentations

Manuscripts:

a) Carvalho, RS, Schaffer, JL, Bumann, A, and Gerstenfeld LC. Integrin Ligands Expressed by Osteoblasts Show Preferential Regulation in Response to Both Cell Adhesion and Mechanical Stimulation. (submitted in 1999 and in revison)

b) Carvalho, RS. Kostenuck, P, Bumann, A, Salih, E,and Gerstenfeld LC Integrin Specific Mediation of Cell Attachment, Second Signal Kinase Activity, and Induction of Osteopontin mRNA Expression. (Manuscript in prepartion)

Absrtacts: None.

Patents: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: None

CONCLUSIONS

The major conclusions from the first year of funding of this proposal are as follows:

1. Integrin ligands as a class are induced in osteoblasts in response to mechanical stimulation and adhesion. Such findings provide strong evidence to support the hypothesis that these molecules act like autocrine or paracrine factors.

2. Cell adhesion of osteoblasts is specifically mediated by β1 class of integrins. This same class of integrins appears to be responsible for the signal transduction process that stimulates osteopontin induction. The β3 integrin ligands vitronectin and osteopontin neither mediate specific adhesion or induce osteopontin gene expression, but they do selectively induce specific kinase activities.

3. Component aspects of the mechanical stimulation do effect the induction of the gene. Both duration and increasing frequency clearly appear to increase response to the mechanical signal. Shear stress induced by fluid flow does not appear to be a mediating factor in osteopontin induction in response to mechanical stimulation.

REFERENCES:

1. Jilka RL, Manologas SC 1994 In *Osteoporosis* Chapter 2 The cellular and biochemical basis of bone remodeling. pg17-47 ed. R. Marcus, Blackwell Scientific Publications, Cambridge MA

2. Lanyon LE, Goodship AE, Pye C, McFie H 1982 Mechanically adaptive bone remodeling. Biomechanics **17**: 897-906.

3. Lanyon LE, Rubin CT 1987 Functional strain on bone tissue as an objective and controlling stimulus for adaptive bone remodeling. J Biomech **15**:141-154.

4. Mori S, Burr DB 1993 Increased intracortical remodeling following fatigue damage. Bone **14**:103-109.

5. Gerstenfeld LC 1999 Editorial\Perspective Osteopontin in skeletal tissue homeostasis: an emerging picture of the autocrine\paracrine functions of the extracellular matrix. J Bone Miner Res **14**:850-855.

6 Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, Duncan RL 1997 Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain . Am J Physiol ;273:C810

APPENDICES

None

Boston University
Medical Campus

Office of Research
Administration
715 Albany Street, 560
Boston, Massachusetts
02118-2394
617 638-4600

BOSTON UNIVERSITY MEDICAL CENTER

Institutional Biosafety Committee

Renewal Letter: Biohazard Project

IBC Coordinator: Mary Gistis

Principal Investigator:	Dr. Louis Gerstenfeld
Project Title:	Musculoskeletal Research
Approval Number:	# A-168
Renewal Date:	November 4, 1998
Containment Level:	BL-2/Universal Precautions
Comments:	related to rDNA projects 497, 500, 501

For your records, make several copies of this document to avoid delays when filing your grant applications with federal agencies.

IBC APPROVAL LETTER

Boston University
School of Medicine

Institutional Animal
Care and Use
Committee

700 Albany Street, W707
Boston, Massachusetts
02118
TEL: 617 638-4263
FAX: 617 638-4055

Louis C. Gerstenfeld PhD
Associate Professor
Orthopedic Surgery
Boston University Medical Center
715 Albany Street, Housman 2
Boston MA 02118

7/11/2000

RE: Application No. 98-096 Agency: Department of the Army

Title: Mechanisms of Mechano-Transduction Within Osteoblasts

Protocol Status: APPROVED, 7/22/1998
ANIMAL NUMBERS/YR. 1248 chicken embryos/year x 4 yrs

BIOHAZARDS:

Dear Dr. Gerstenfeld

Your application for use of animals in research or education has been reviewed by the Institutional Animal Care and Use Committee at Boston University Medical Center. The protocol is APPROVED as being consistent with humane treatment of laboratory animals and with standards set forth in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

The Laboratory Animal Science Center at Boston University Medical Center has been accredited by the American Association for Accreditation of Laboratory Animal Care since 1971. Boston University Medical Center has had an Animal Welfare Assurance on file with the Office for Protection from Research Risks (OPRR) since January 1, 1986. Boston University's Animal Welfare Assurance number is A-3316-01.

Animal protocols may be approved for up to three years. However, if the study extends beyond one year from the approval date, an annual continuation form (1 page) must be submitted. If a project is to extend beyond three years, a full application must be resubmitted and reviewed at the end of the initial three year period.

Sincerely,

Colleen A. Cody, Coordinator
Institutional Animal Care and Use Committee

c: Wayne W. LaMorte, M.D., Ph.D., M.P.H. Chairman, IACUC
Veterinary Staff, LASC

BOSTON UNIVERSITY MEDICAL CENTER

Radiation Protection
Office

88 East Newton Street, D-604
Boston, Massachusetts
02118-2394
617 638-7052

To: Dr. Gerstenfeld

Date: June 24, 1998

From: Victor Evdokimoff, Secretary Radioisotope Committee VNE/CM

Subject: Authorization to use radioisotopes at BUMC

On June 24, 1998 your application _X_, renewal ___, amendment____ to use radioisotopes at BUMC was approved. You are only authorized for the following isotope(s), quantities, etc.

Isotope(s)	Form	Max./order	Max./year	Possession limit
H-3	Amino acid, Nucleotide	5 mCi	N/A	N/A
P-32	Phosphorous, Nucleotide	5 mCi	N/A	N/A
C-14	Chloramphenicol, Amino acids	1 mCi	N/A	N/A
S-35	Na Sulfate, Nucleotide, Amino acids	1 mCi	N/A	N/A
P-33	Phosphorous, Nucleotides	1 mCi	N/A	N/A

Your authorization code number is G-16. This number must appear on all requisitions for isotopes. In addition, the following conditions apply to your authorization:

1) The Radioisotope Committee is recommending you consider alternatives to using 3H/14C such as non-radioactive tracers.

2) All personnel under your permit planning on using P-32 must complete the individual training requirements. In addition, any user of 1 mCi or more of P-32 at one time is required to be monitored by TLD. Please contact the RPO for assistance.